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APPLICATION OF CABIN ATMOSPHERE MONITORS TO RAPID SCREENING OF BREATH SAMPLES FOR THE EARLY DETECTION OF DISEASE STATES

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PREFACE

The Department of Physics in conjunction with the Schools of Pharmacy and Medicine of the University of Missouri - Kansas City is conducting research regarding the application of human breath analysis to the analysis of physiological states. These applications to health are the outgrowth of technological developments sponsored by the National Aeronautics and Space Administration.

Dr. Paul J. Bryant of the Department of Physics and Dr. Jimmie L. Valentine of the School of Pharmacy have served as Co-Investigators. Research work was completed in the Mass Spectrometry Laboratory under the direction of Mr. Paul L. Gutshall. Mr. Owen Gan, Mr. Everett Thompson and Mrs. Patricia Lovegreen have conducted research involving high pressure chromatography and gas chromatography in the School of Medicine and Pharmacy under the direction of Dr. Valentine.

SUMMARY

Analysis of human breath is a simple, convenient and most important, a non-intrusive method to monitor both endogenous and exogenous chemicals found in the body. The present study has investigated and developed several technologies which are applicable to monitoring some organic molecules important in both physiological and pathological states. Two methods were developed for enriching the organic molecules exhaled in the breath of humans. One device is based on a respiratory face mask fitted with a polyethylene foam wafer; while the other device is a cryogenic trap utilizing an organic solvent. Using laboratory workers as controls, two organic molecules which occurred in the enriched breath of all subjects were tentatively identified as lactic acid and contisol. The amounts of lactic acid varied on the breath during the test period whereas cortisol appeared to be rather constant. Variation in the extent of muscle activity may have been responsibile for these observed differences. Both of these substances occurred in breath in sufficient amounts that the conventional method of gas-liquid chromatography was adequate for detection and quantification.

To detect and quantitate trace amounts of chemicals in breath, another type of technology was developed. Again the breath was enriched in organic compounds using the breath collection devices, but analysis was conducted using high pressure liquid chromatography and mass spectrometry. By using a stable isotope and peak matching, one nanogram of a chemical could be detected and quantitated -- using the electron impact mode of the mass spectrometer. Alternately, direct assays of the breath pattern could be obtained using field ionization mass spectrometry.

The developed technologies for assaying human breath and the limited applications thereof, appear suitable for monitoring certian physiological parameters in humans. Particularly is this true when a metabolic product generated during a physiological process is detectable in breath, for example lactic acid. Also, the technology has been shown to be practical for detecting metabolic products generated by bacteria. Precise monitoring of breath by the developed technology would allow for early warning of an impending bacterial infection. Such an application has been studies for streptococcus bacteria.

CONTENTS

INTR DUCTION

Human breath has for numerous years been recognized as containing volatile constituents other than the common respiratory gases. Ever since fermentation of fruits to produce alcohol was discovered until now, people have detected the characteristic odors of ethyl alcohol and its metabolites on the breath of a person who has consumed an alcoholic beverage. With the advent of modern instrumentation, it has become possible to scientifically document the presence of ethyl alcohol and its metabolites on human breath as well as other volatile constituents. Several research groups (1,2) have recently demonstrated the presence of many volatile constituents in normal human breath using gas liquid chromatography (glc). Similarly, glc has also been used to identify such compounds as acetone (3), alcohols (4), aldehydes (5), anesthetics (6), fatty acids (7), hydrogen (8), mercaptans (9) and methane (10) in human breath. Some of these compounds appear in greater quantities in human breath during pathological states and all are present in such large quantities in expired ir that no enrichment techniques are required.

Combined gas liquid chromatography - mass spectrometry (glc-ms) has been used to detect and in some cases quantitate ethanol and other low molecular weight compounds of biomedical interest when found in human breath (11). One report has appeared (12) which describes the use of field ionization mass spectrometry (ms) to detect low molecular weight alcohols in human breath. Another report (13) detailed the enrichment of human breath using a multi-stage separator followed by ms analysis which was capable of detecting small amounts of several organic compounds introduced into the body.

The present study was designed to further develop instrumental methods and techniques for detecting and quantifying chemical substances which might be present in human breath. An initial hypothesis was made that a compound which was to be measured would be present in exceedingly small amounts. Such a belief was based on the fact that in the normal human male about 0.5 liter of air is expired in a complete expiration of breath (14). Thus some device capable of enriching the expired air stream for the compounds of interest was felt to be absolutely essential. Two such enrichment devices have been designed and tested for their utility in enriching expired air.

Considerable effort was also expended in the present work in developing instrumental techniques which could detect small amounts of compounds present in the enriched breath samples. Development of such instrumental methods had to meet three strict criteria, viz., specificity, reproducibility and sensitivity. Several chromatographic - mass spectrometry methods have been devised and applied to the analysis of trace constituents in human breath.

In an effort to correlate the applicability of the developed breath analysis techniques to the monitoring of individuals with disease states, a laboratory study was also conducted. This study included the identification of the chemical by-products of several bacteria and one fungus. Emphasis in this study was placed upon determining the difference between normal blood and blood which had been incubated with the organism of interest. Extraction and instrumental techniques have been developed for accomplishing these limited objectives. The reason for studying these methods is that humans are frequently infected with either bacterial or fungal organisms. The intermediary metabolism of these organisms is often quite different than that of its human host. For example, the glycolysis pathway in both bacteria and fungi is different than the same pathway in humans. In most cases these organisms produce certain metabolic by-products which are sufficient to characterize

them. That is one strain of bacteria may produce large amounts of propionic, pentanoic and olelic acids, whereas no other strain would produce this exact combination. Thus it is possible for each organism to give a "signature" to its species type via its metabolic products. With the knowledge that most of these metabolic products are relatively low molecular weight compounds, the supposition was made that they might appear as volatile substances in expired breath. However, prior to human studies it was felt that some preliminary work on *in vitro* human blood systems which were impregnated with a bacterial or fungal organism would be necessary. These studies have been conducted and are reported herein.

EXPERIMENTAL METHODS

A. Methods of Breath Analysis

One objective in the present study was to devise methods for enriching the expired air of a human. Since the chemicals indicative of a physiological or pathological state would most likely be present in small amounts, it seemed reasonable that a repeated enrichment of the expired air stream could produce amounts amenable to instrumental analysis. Two different enrichment methods were devised and evaluated as discussed below.

1. Polyethylene Foam Wafer

Several years ago the Bendix Corporation in Kansas City developed a process for producing polyethylene which contained varying pore sizes (15). The size pore was adjusted by using sized NaCl crystals during the molding process. These salt crystals were removed by allowing the polyethylene to stand in water over approximately a one week period of time. Experimental results indicated that the pore size produced by unsized table salt gave the least resistance to expired air. The polyethylene foam wafers used in the

human experiments described later were cut so as to give a disc 3.0 cm in diameter and 0.25 cm thick. Further results from the clinical trials indicated that the hot molded polyethylene wafers were most practical because of their mechanical strength and durability. Figure 1 illustrates a cross-sectional view of a polyethylene foam wafer. In theory this wafer should allow small airborne gases such as oxygen, nitrogen, sulfur dioxide, certain hydrocarbons, etc., to pass through while retaining the larger organic molecules, such as Δ^9-tetrahydrocannabinol (THC). Earlier work (16) in our laboratories has shown that THC will bind to the polyethylene foam wafer. The exact mechanism of this binding is not known but it could simply be a physical phenomena due to the tortuous pathway enhanced by the induced dipole bonding mechanisms i.e., Van der Waals attraction. In the case of THC as well as other organics, this binding is reversible when the wafer is extracted with an organic solvent with a greater affinity for the molecule.

To position the polyethylene foam wafer in front of the expired air stream, a rather simple device was devised to hold the wafer. This device consisted of a standard canister respirator mask (Welch model 7500-30G) which was modified by placing a 2.5 cm diameter hole directly in front of the mouth. On the interior of the mask was placed a 4.5 cm (o.d.) by 2.0 cm thick rubber ring of sufficient flexibility to firmly hold the polyethylene foam wafer. Thus the foam wafer was held directly in front of the subject's mouth and approximately 1.5 cm away from the lips. In addition a small flapper valve was placed over the outer hole so as to prevent inhaled air from being drawn back through the wafer. Figure 2 gives a cross sectional view

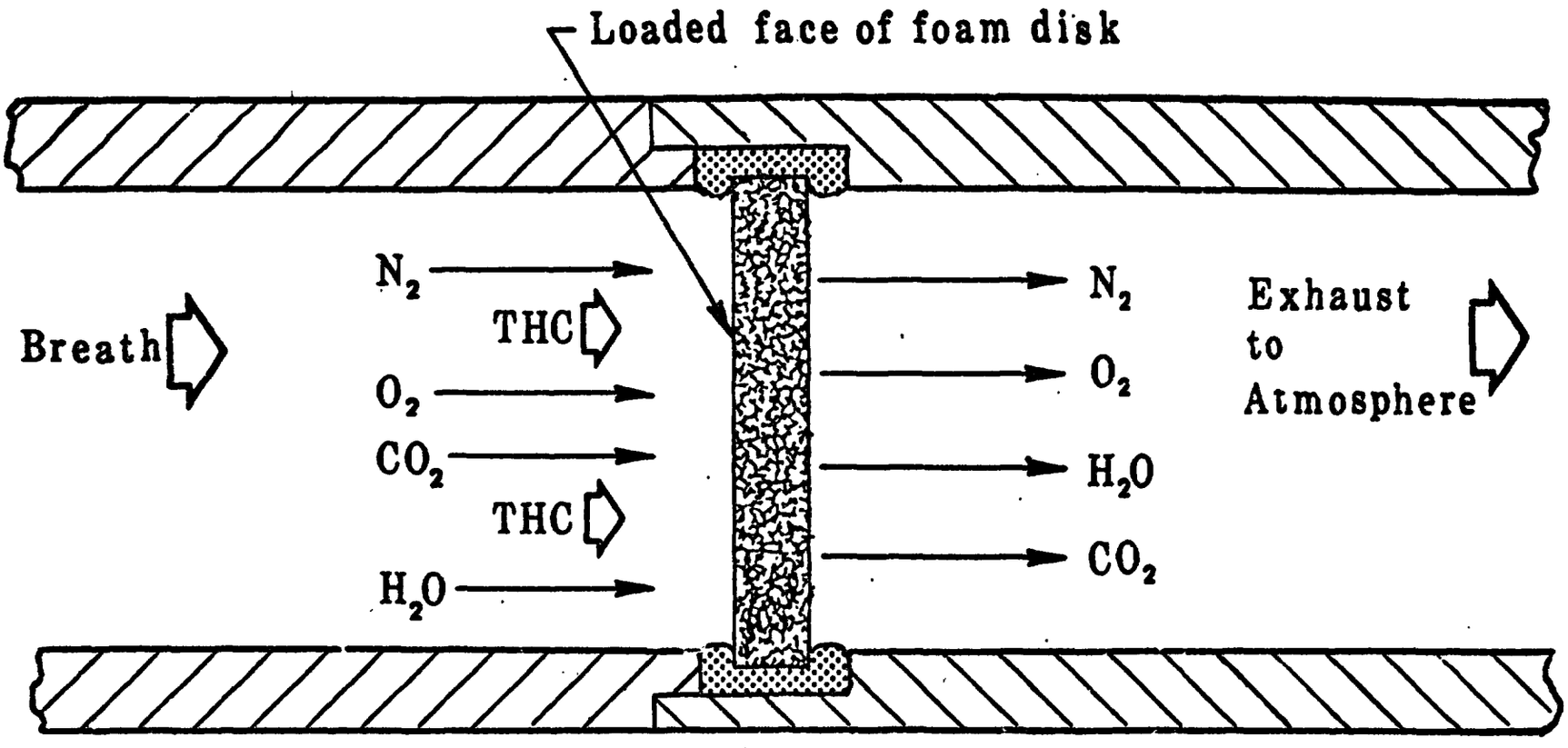


FIGURE 1

Cross-Sectional View of the Polyethylene Foam Wafer For Breath Collection

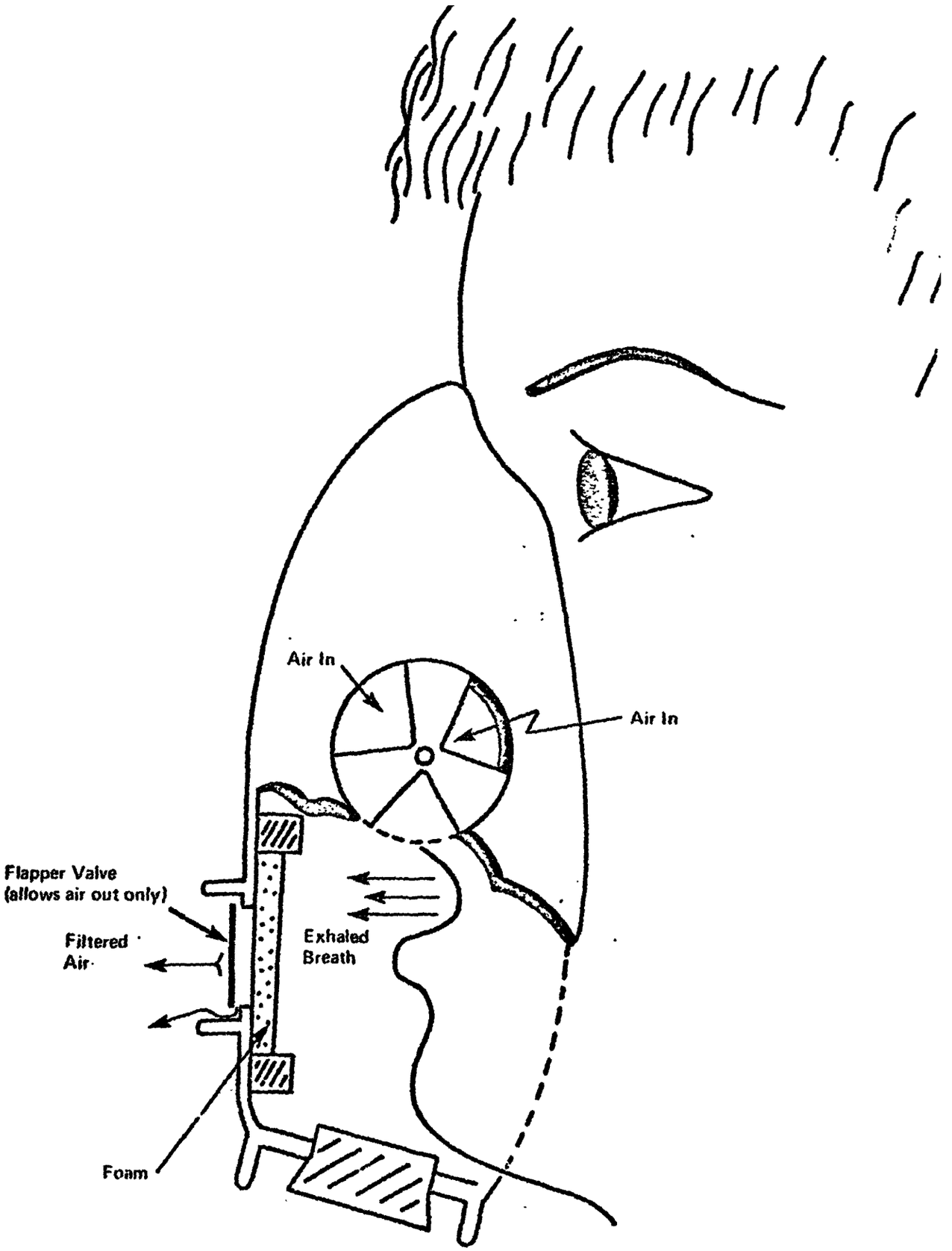


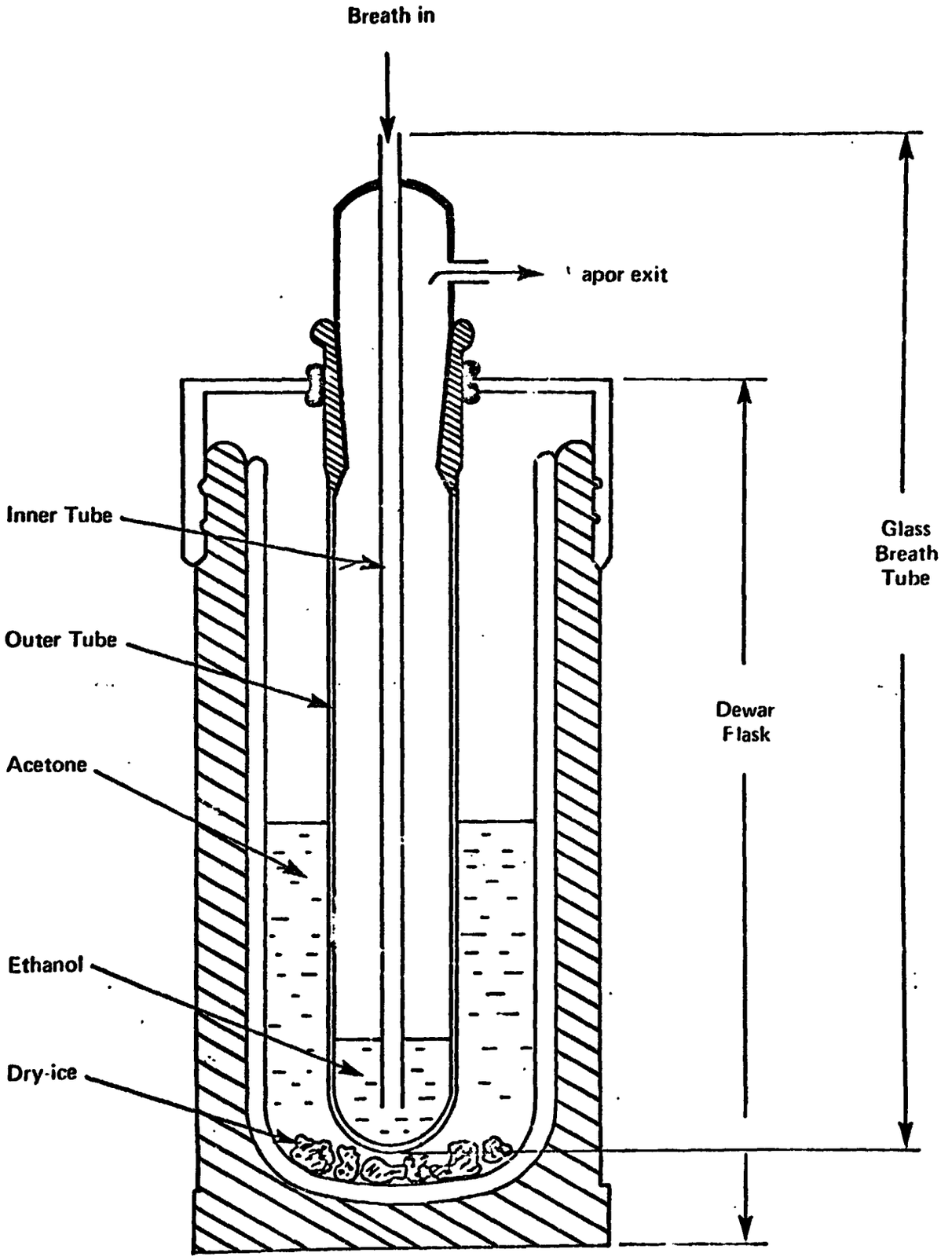
FIGURE 2

Cross-Sectional View of Face Mask
Containing the Polyethylene Foam Wafer

of this modified mask. To obtain an enriched sample of expired air each subject was asked to breathe for one minute with the mask positioned over the nose and mouth as shown in Figure 2 using deep inhalations and exhalations through the mouth. The polyethylene foam wafers were placed in and taken from the mask using disposable examination gloves. If the wafers were to be stored for a certain time period prior to analysis, they were placed in small silinized petri dishes. Each wafer was prepared for analysis by subjecting it to 30 minutes of ultrasonication while submerged in 10 ml of methanol in a slinized 50 ml beaker. Upon completion of sonication, the methanol was decanted into a siiinized test tube followed by evaporation to dryness under nitrogen. The residue was reconstituted in an appropriate solvent depending upon the analysis method to be used, viz., glc or hplc. Both analysis methods are described in a later section.

2. Ethanol Breath Tube

Earlier workers (1) in the field of breath analysis had successfully used a "cold trap" to condense airborne breath particles. Since most organic volatiles carried on breath would be soluble in organic solvents, an apparatus was designed which used both an organic solvent and a cold trap. A number of different designs and configurations were tested but the one which proved to be most applicable is shown in Figure 3. This apparatus will be referred to as the "ethanol breath tube" since most of the breath analysis work reported for this technique utilized ethanol as the organic solvent. However, as will be pointed out in a later section, the organic solvent can be varied to "trap" a particular substance in breath. Therefore, by using an appropriate solvent and cold temperatures the extraction of



ETHANOL BREATH APPARATUS

FIGURE 3

organics from the breath as well as thermally condensable volatiles is maximized.

The breath trap shown in Figure 3 consists of a re-entrant cylindrically shaped glass trap, with a center tube of 15 mm diameter while the outer tube has a diameter of 25 mm. Each subject is asked to exhale their breath as completely as possible down the inner tube. In so doing the breath passes through the annular space between the inner and outer tubes. The well volume in the bottom of the trap is filled with 10 ml of ethyl alcohol, or some other appropriate organic solvent. A cryogenic condensation is applied by submerging the trap into a dewar containing a dry ice-acetone mixture. In general, trace components can be extracted from two tidal volumes of expired breath.

B. Methods of Instrumental Analysis

Analysis of the breath samples taken via one of the above methods required different procedures depending upon amounts of chemical substances present in the expired air. For example, if microgram quantities of a chemical were present, the common detector systems of glc and hplc, viz., flame ionization and uv spectrophotometer, respectively, were sufficiently sensitive. However, for trace substances, i.e., less than 1 microgram, the high sensitivity of the mass spectrometer was required. Yet, even when the mass spectrometer was used the separation ability of the chromatographic methods was utilized to achieve the needed selectivity. All of these developed techniques are described below.

1. Gas-Liquid Chromatography

All glc determinations were performed using a Varian 2740 gas chromatograph equipped with an automatic linear temperature programmer, dual electrometer and dual hydrogen flame ionization detectors. Each sample was analyzed on two different stainless steel columns. One column was a 0.92 m (3 ft.) X 1.0 cm 3% SE-30 on 80/100 Chromosorb WH-P and the other a 1.84 m (6 ft.) X 1.0 cm 3% OV-17 on 80/100 Chromosorb WH-P. Gas flow rates were: air 300 ml/min, hydrogen 30 ml/min and nitrogen (carrier gas) 30 ml/min. Each sample was analyzed using a temperature program from 60-260°C with an initial 2 min. isothermal hold and 5 min. post hold. Temperature rise was 10°/min. and most determinations were made using electrometer settings of range, 10^{-11} and attenuation, 32.

To the residue obtained from the evaporation of the methanol extract from the polyethylene foam or the organic solvent from the breath tube was added 30 µl of N,O-bistrimethylsilylacetamide (BSA, Pierce Chemical Company). A 4 µl aliquoit of the resultant solution was injected into each column described above. For glc analysis of the blood extracts described later, a different silinization procedure was used. To the residue was added 1.5 ml of pyridine, 0.3 ml hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. This solution was throughly mixed for 1 minute (on a Vortex Mixer™) and allowed to stand at room temperature for 15 min. A separation of layers occurred which could be enhanced by centrifuging at 3000 rpm for 15 min. The top layer is removed and evaporated to dryness at room temperature and the residue reconstitued in 300 µl of ethyl ether. A 3 µl aliquoit is analyzed by glc.

2. High Pressure Liquid Chromatography

All hplc analyses were performed on a Varian 8520 gradient elution liquid chromatograph utilizing a Varian 635M recording spectrophotometer set at 273 mμ as a detector. The column was a Varian Si-10 (10 μ silica gel), 25 cm X 2 mm (i.d.). For the breath analyses, a gradient elution program was developed which started at 95:5, heptane:methylene chloride and proceded to 95:5, methylene chloride: heptane over a 9 min. period. The program is then reversed, i.e., from 95:5, methylene chloride:heptane to the initial 95:5, heptane:methylene chloirde, thereby regenerating the column. A solvent flow rate of 120 ml/hr was used for all determinations. To facilitate collection of the effluent droplets eminating from the hplc a 10 cm "zero dead volume" stainless steel tube was attached to the flow cell of the spectrophotometer.

The residue obtained by evaporating the methanol extract from the polyethylene foam or the orgainc solvent from the breath tube is reconstitued in 300 μl of heptane. Injection of the 300 μl solution onto the hplc is followed by a 200 μl heptane wash. The gradient is begun and samples collected as peaks appear on the hplc chromatogram. Since the instrument has stop flow capabilities, a peak of interest can be scanned over the uv-visible spectrum.

3. Mass Spectrometry

Two major technologies involving mass spectrometry have been developed and applied to human breath analysis. Actual samples from patients in known physiological states have been analyzed. Both qualitative and quantitative assay techniques were developed.

The first mass spectrometry procedure for human breath assays utilized field ionization. Due to the lack of fragmentation of molecules in this procedure it was possible to observe a full breath spectrum in one assay. Thus, a multi-component analysis can be performed rapidly by this field ionization mass spectrometry technique. The total time between the breath sampling and the production of a multi-component data plot is less than 30 minutes. A rapid multi-component assay is desireable for some applications, such as screening tests for a number of different compounds on human breath (12).

A new technology has been developed to compliment the multi-component assay technique. This new methodology provides very high sensitivity and specificity for individual components of human breath. Sensitivity is raised greatly by directing the mass spectrometer to the specific compound for a timed recording period rather than scanning over that peak rapidly as is done in an assay. Specificity is increased by applying liquid chromatography for separating of the component of interest before introduction to the mass spectrometer.

The liquid chromatography portion of the new technology required a conversion of the breath sample to a liquid form. Two different methods were designed and tested for the collection and concentration of breath components into a liquid form as described in the Experimental Methods section of this report. Each of the mass spectrometry techniques, outlined above, will be described in more detail below.

a. Field-Ion Mass Spectrometry

The use of field ionization in place of electron impact ionization has been developed and applied by our laboratories for several years under NASA sponsorship. The reports (17,18) from these programs describe the detailed design of a special graphite filament ion source which produces parent ions without the normal cracking or fragmentation which occurs when electron-impact ionization is employed. The details of the design and various applications are fully described in the reports and publications referred to in references 12 and 18. It will surfice here to mention the overall characteristics of the field-ion breath spectrum obtained when the graphite filament field ionizer is employed. The spectrum consists of parent molecular ions representing the molecular components of that individuals breath sample. Thus, the technique does provide a multi-component analysis of a sample.

There can be some question regarding the specificity of the individual mass peaks which constitute the spectrum, since only one peak occurs for each component. The question would involve the possibility that more than one compound could produce the same peak. Since high resolution mass spectrometry is being employed, this question is not too serious. However, for the detection of some trace substances, wherein the maximum sensitivity is required, it is not desireable to use high resolution. When the combination of lower resolution and the resulting higher sensitivity values are employed, there is a question about the identification of a trace compound. To compliment the field ionization technique in such situations, an alternate method was applied as described below.

b. High Pressure Liquid Chromatography - Mass Spectrometry (hplc-ms)

The combination of liquid chromatography to separate desired compounds from the bulk of a sample before introduction to standard electron impact ionization mass spectrometry has been very successful. The application of this combined technology to breath samples has been even more successful than expected.

The combination referred to above includes the extraction of breath components from the polyethylene foam wafers by an appropriate liquid organic solvent, followed by hplc separation and ms assay. This technology is not as rapid as the direct field ion assay of breath samples. However, when the latter (field-ion) technique is employed as a rapid screening assay followed by the hplc-ms analysis of an individual compound of interest, a powerful assay technology is realized.

A more detailed description of this technique is as follows. The extraction of breath components was described above. A one milliliter portion of hplc effluent is collected in a silinized vessel, then either stored at low temperature for later analysis by ms or immediately evaporated to near dryness with a dry nitrogen stream. The concentrated effluent is then loaded into a 5 microliter gold cup by means of a micro-syringe. The remaining solvent dries quickly; the gold cup is then inserted into the ion source region of the mass spectrometer via a direct insertion probe.

The ms analysis employing the standard electron impact technique may be conducted since the bulk of the components of the sample have been separated by the chromatography procedure. This combined hplc-ms analysis may be used to advantage either following a multi-component assay by the field-ion screening method or it may be employed to detect a suspected component directly from a breath sample.

c. A Quantitative Assay Technology Employing hplc-ms

The further combination of hplc-ms and the peak matching ion counting technologies have produced a quantitative mass spectrometry capability. This capability has now been designed, developed and applied to a large number of patients with both known and suspected levels of drug toxicity.

This quantitation is most readily applied to body fluid samples since the quantity of a substance per milliliter of plasma or saliva may be reported. Whereas the quantity of a breath sample is more nebulus. However, this quantitation method has been successfully applied to equal volumes of breath.

The quantitative mass spectrometry technology is as follows. A known amount of a stable isotope of the compound of interest is added to the liquid extract of either the body fluid or breath sample. This sample is then passed through hplc without appreciable separation of the suspected compound and its stable isotope. The effluent droplets, containing the compound of interest, is processed into the mass spectrometer as described above. In this case, for a quantitative measurement and a positive detection of the suspected compound, the peak matching ability of the ms is utilized. That is, the ms is focused on the center line of the stable isotope peak, which is present in abundance, and the known mass ratio is dialed into the peak matching accessory. The ms is then directed to the center line of the suspected compound. The major fragment peaks may be similarly checked for positive identification of the substance.

For trace quantities of substances, i.e. for low nanogram or picogram amounts, this technology provides sufficient sensitivity for both positive identification and for quantification. In the case of a trace substance, a peak will not be directly detectable on an oscilloscope screen or on a chart recorder. To increase sensitivity by approximately three orders of magnitude an ion counter integrator has been employed along with a peak height discriminator. Each individual ion which strikes the first dynode of the multiplier detector in the ms causes a count to be registered in the integrator storage register. Furthermore, by this peak matching - ion counting combination, it is possible to extend the time for integration of the signal until the entire breath (or body fluid) sample has been depleted. The depletion process does not alter the quantitation since a multi-point comparison is carried on between the compound being quantitated and the known amount of its isotope. That is, by setting the peak matching to a relatively rapid switching time the alternate comparisons are made without significant effect due to longer range sample depletion.

The switching time usually employed is 67 milliseconds. Thus the ion counter integrator stores counts in one register from the compound for 67 milliseconds, then it stores counts in a second register for 67 milliseconds from the stable isotope of the compound. After 1000 counting periods or a total elapsed time of 67 seconds, the sample is nearing depletion. The total counts in the two registers yield a quantitative assay of the trace compound under investigation.

C. In Vitro Human Blood Studies

For the following study, human blood plasma obtained from either blood bank samples or volunteers was used. The plasma was obtained by centrifuging whole blood for 20 minutes at 2600 rpm. Plasma was then filtered through a 0.41 micron Milli-Pore™ filter and stored under aseptic conditions. The plasma was sub-divided into two portions. One part was used as the control in the subsequent analyses, whereas the other half was incubated with the organism of interest. The period of incubation varied depending upon the fungus or bacteria under investigation. All plasma impregnations were done by Dr. Phillip Rhodes and Mrs. Gay Curtis at Children's Mercy Hospital, Kansas City.

1. Studies on Candida Albicans

Method A -- One ml of each the control and infected plasma was mixed with 1 ml of an 8N HCl in methanol solution. Each sample was heated for 1 hr in a water bath maintained at 80°C. To each sample was added enough ammonium hydroxide to obtain pH 7 followed by evaporation to dryness under reduced pressure at 70°C. The residue was then reconstitued and analyzed by glc as described above.

Method B -- One ml of both control and infected plasma was extracted with 1 ml of heptane which had been saturated with phenol. In most cases, centrifuging was necessary to separate the layers. The top layer containing the organic solvent mixture was removed and evaporated to dryness under nitrogen at room temperature. The resultant residue was reconstituted and analyzed by glc as described above. Alternately, to the residue could be added 20 µl of a commercially available Tri-Sil DMF™ solution (Pierce Chemical Company) followed by a 4 µl injection of this material into the gas chromatograph using the conditions described above.

2. Studies on Streptococcus

To 1 ml of both control and infected plasma was added 1 ml of 5% aqueous hydrochloric acid. The mixture was extracted with 5 ml of methylene chloride and centrifuged to effect a separation of layers. The lower organic extract layer was removed and evaporated to dryness at 50°C under nitrogen. The resultant residue was silinized as described in Method B above and analyzed by glc as previously described.

RESULTS AND DISCUSSION

For breath analysis to be effective in delinating a disease state or to give forewarning of one, there must be an adequate baseline established in a non-diseased individual. In the present study, therefore, one objective was to apply the technology developed to a limited number of individuals to determine if such a baseline could be established. For this study five laboratory workers were used. All were in good health with no history of recent illness and taking no medication. Two of the subjects were male and three female, with ages between 25-35 years, average age 27 years. Each subject had their breath analyzed three different times during the test day, 10:00 a.m., 1:00 p.m. and 4:00 p.m. These specific times were chosen because just prior to the 10:00 a.m. sample all of the subjects except one female had drank coffee; prior to the 1:00 p.m. sample all subjects had lunch; and the 4:00 p.m. sample gave an indication of the breath three hours after eating. The breath from each subject was taken at each sampling period utilizing both the face mask containing the polyethylene foam wafer and the breath tube containing ethanol. In all cases the face mask was used first followed by the breath tube five minutes later. Each sample was analyzed by glc as described in the previous section. Table I gives the results obtained from this study.

The results shown in Table I clearly indicate that the face mask containing the polyethylene foam was the most consistent and best method for enriching the expired breath. Also a quite surprising finding was that unlike other findings (1,2) these results were not extremely complicated due to a large number of compounds

TABLE I -- Number of Peaks Observed From the glc Analysis of Breath Enriched by Two Methods.

Subject Number	Sex	METHOD OF ENRICHMENT					
		Polyethylene Foam			Breath Tube		
		10:00 am	1:00 pm	4:00 pm	10:00 am	1:00 pm	4:00 pm
1	M	3	3	3	1	2	1
2	M	3	3	3	2	2	2
3	F	3	3	3	1	2	2
4	F	3	3	3	2	2	3
5	F	3	3	3	1	1	1

(peaks) appearing in the chromatogram. Thus, it appears that the newly developed method of breath collecting, especially the polyethylene foam wafer, does allow most of the hydrocarbons on breath to selectively pass through while retaining the polar organics. The fact that polar organics do bind to the polyethylene is strengthened by the tenative identification of two compounds on the breath of the study subjects as polar compounds. Based on retention times of authentic samples the peaks at 3.8 and 8.8 min have been identified as lactic acid and cortisol, respectively. The third peak which occurred at 10.1 min has not been identified. This limited study was not designed to quantitate the substances found on breath but rather to determine a pattern for a healthy individual. However, the observation was made that the relative peak areas for the three substances on breath did vary with the time of day in each individual. The most dramatic increase occurred in the amount of

lactic acid and may be attributable to the extent of muscle activity prior to the given breath sample.

The experiments performed with the human blood plasma impregnated with Candida albicans and streptococcus were a limited success. The work with Candida was prompted by the report of Miller, et al. (19) in which they reported a qualitative glc difference between the blood serum of patients with Candida albican septicemia and control serum. These authors concluded that the presence of mannose in the infected serum was the major difference. A number of experiments in our laboratory failed to reproduce their results. Furthermore serum to which mannose had been added failed to produce the type of chromatogram reported by these authors. Therefore, an alternate extraction method was devised, viz. Method B, which would quantitatively extract the mannose from blood plasma or serum to which it had been added. When this method was applied to plasma which had been impregnated with Candida, no peaks which corresponded to mannose were noted. This experiment was repeated with three different plasmas infected with Candida at three different times. Only during glc analysis of one sample was there a distinct difference between the chromatograms of control and infected plasma. Thus the results from this study were indecisive. The findings with the streptococcus were somewhat better. This experiment was also performed on three different plasmas which had been impregnated with the streptococcus. In the work, the plasma was extracted at acid, base and ambient pH, then analyzed by glc. Best results, that is differences between control and infected plasmas, were noted in the acid extract, most likely because this bacteria is the gram-positive type. The difference in each sample was apparently attributable to lactic acid. That is, the size of the lactic acid peak was markedly increased in the bacteria infected plasma. This procedure has been applied to only one clinical case in which blood plasma of an infant suspected of either a streptococcus or pseudomonous infection was analyzed by the method. No increase in the lactic acid content was noted; yet when the child's blood was cultured, it showed a positive for

streptococcus. The exact reason why the glc procedure failed in this case is not entirely clear. However, it may be that it was simply a problem with sensitivity, i.e., the lactic acid may have been present but was not detected due to the very small amount of blood analyzed (0.5 ml). All of the experimental work had been developed using 1 ml of plasma.

Although the glc procedure may not have been sensitive enough to have detected other low molecular weight acids, they could have been present. For example butyric and propionic acid, which are commonly produced by gram-positive bacteria most likely [illegible] some of the other acids present.

For development of the technology capable of detecting such metabolic by-products if they appeared on breath, a series of experiments utilizing ms were conducted. First a series of experiments were conducted using the polyethylene foam material to which was added known amounts of acetic, propionic, butyric, pentanoic and lactic acids and also butanediol. The foam material could be extracted with an organic solvent then analyzed by ms, but the more convenient method was to introduce a small portion of the foam to the ms via the direct insertion probe. Detection of the acids was achieved using both electron impact (monitoring the most abundant ion) and field ionization sources. Either of these sources was capable of detecting 0.5-1 microgram of material. To detect nanogram and picogram amounts of these acids it is necessary to use a stable isotope of the known acid and peak match using the ion counting technique. This technique is readily applicable to most of these low molecular weight acids since the deuterated form of these are commercially available (Merck Isotope Company or Aldrich Chemical Company). For example, propionic acid was quantitatively determined by adding 1 µg of d_3-propionic acid (mass 77) to the foam and focusing on this mass then stepping to mass 74 using the peak matching accessory. This switching occurs many times as described earlier to give an accurate statistical correlation and

quantitative value for the undeuterated propionic acid. This technology has not been applied to a large number of breath samples. However, several polyethylene foams which were loaded with a subject's breath sample then had a known amount of propionic acid added. The polyethylene foam was analyzed successfully by ms using moderate resolution (ca. 1000) without prior chromatography. If a larger number of breath samples were evaluated, a more definitive evaluation of possible interferences which could require chromatography might be made.

CONCLUSIONS

A general objective of the present study was to further develop technology for analyzing human breath and in particular find "biochemical markers" which might give an early warning of an impending disease state. To achieve this broad objective, technology has been developed which first allows for an enrichment of trace substances found in expired breath and second permits determination of these substances in the picogram to microgram range.

Using this newly developed technology, it has been possible to establish that three chemicals occur routinely on the breath of a small group of healthy volunteers. Two of the substances have been tentatively identified as lactic acid and cortisol. The levels of lactic acid on the breath appear to fluctuate; whereas the level of cortisol was fairly constant. Such a finding suggests that one of the routes of excretion, *viz.* the lungs, is used to eliminate some of the lactic acid produced during periods of increased muscle activity. Also the mineralocorticoid, cortisol, must be remaining constant on the breath since mineral content of the body has not changed. The limited scope of the present study has precluded a positive determination of the exact mechanisms operating in the excretion of these tw substances. However, future studies could be designed which would permit a correlation between lactic

acid excretion and the extent of muscle activity and between levels of corticol and electrolytes secreted on breath. The latter correlation would be particularly useful in understanding the relationship between cortisol and calcium loss from the body at zero gravity.

Results from the in vitro human blood studies have given a positive indication that the bacteria streptococcus produces a clearly detectable "biochemical marker." Work with the polyethylene foam to which known amounts of these potential "markers" have been added has shown that mass spectrometry has the sensitivity to detect picogram amounts. A certain number of these "markers" can be identified by mass spectrometry without prior chromatography whereas in some cases chromatography is required to effect the necessary selectivity. But in either case, the technology has now been developed and needs to be applied to a clinical situation for further refinement.

An extremely important finding from the present study as well as other studies done in our laboratories is that a relatively large organic molecule can be found in human breath. Prior to this work a number of workers had shown that the smaller molecular weight compounds, i.e., volatile compounds, are found on human breath. But the finding of cortisol, molecular weight 362 and our prior finding of Δ^9-tetrahydrocannabinol, molecular weight 314, on human breath demonstrates that large molecules can also be expired from the lungs.

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